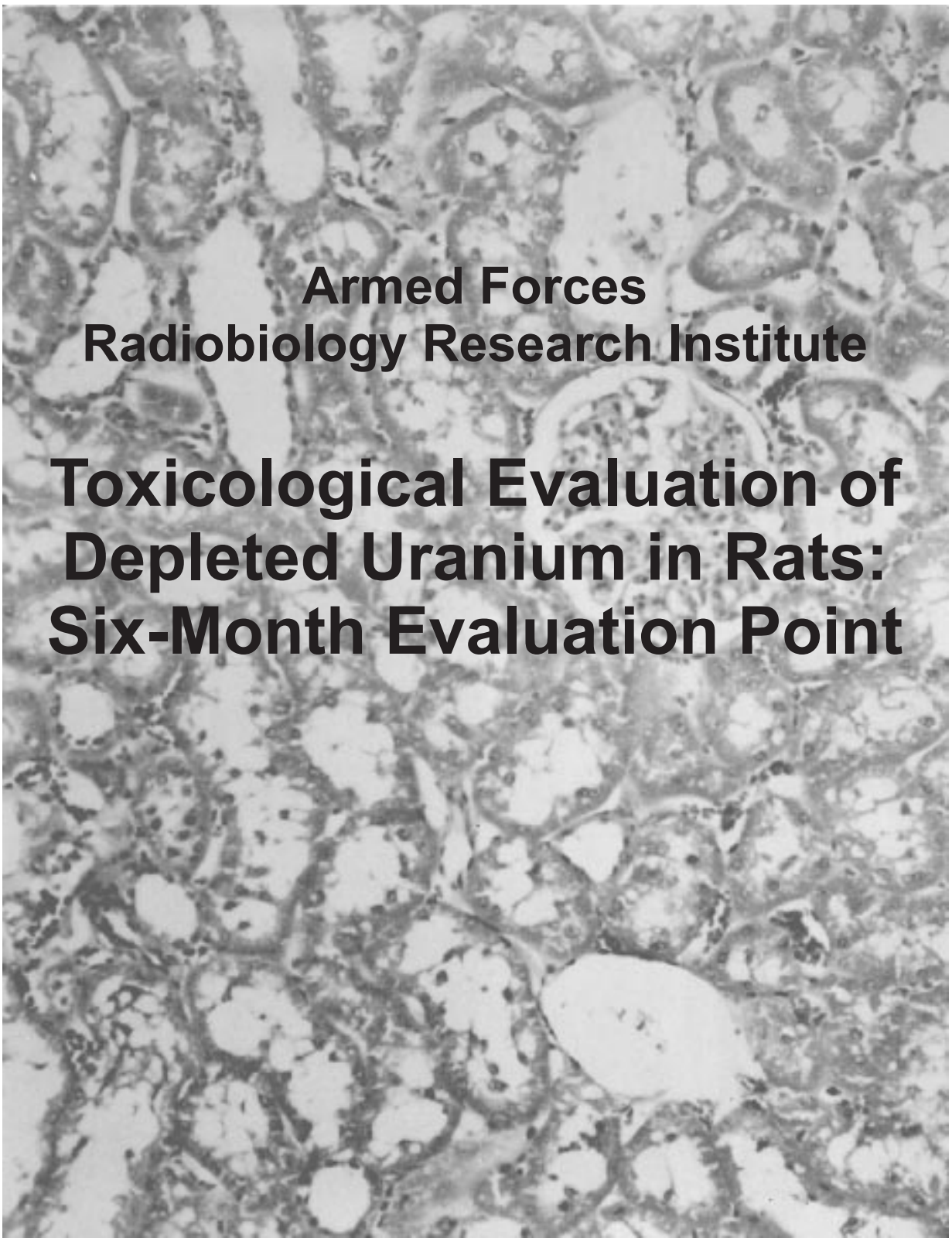

Armed Forces
Radiobiology Research Institute

Toxicological Evaluation of Depleted Uranium in Rats: Six-Month Evaluation Point

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Contents

Abstract

The use of depleted uranium (DU) munitions during Desert Storm resulted in a unique type of battlefield casualty, DU shrapnel wounds. The toxicity associated with embedded DU may differ significantly from other metals or other routes of uranium administration. This is a 6-month interim report of an 18-month study that is designed to assess the toxicity of implanted DU pellets. This study evaluates kidney, behavioral, and neural toxicity associated with intramuscularly implanted DU pellets (1-mm x 2-mm) and assesses tissues for histological changes and for uranium content. Rats were assigned to five experimental groups: 1) a non-implanted sham-surgical control group, 2) rats implanted with 20 tantalum (Ta) to control for fragment implantation, 3) rats implanted with low-dose DU (4 DU and 16 Ta pellets), 4) rats implanted with medium-dose DU (10 DU and 10 Ta pellets), and 5) rats implanted with high-dose DU (20 DU pellets). Uranium levels were high and dose-dependent in the kidney, urine, and bone. Despite high uranium levels in the kidney, no renal toxicity was evident. Between 23–26 weeks body weight in high-DU dose animals was significantly lower than controls. Unexpectedly, uranium was found in the brain of DU-implanted animals. No behavioral neurotoxicity was evident. Excitability of hippocampal neurons was reduced in the high DU dose animals at 6 months. These data suggest that at the 6-month time point, renal toxicity may be less of a hazard than anticipated.While these results indicate that toxicity is not evident at 6 months with exposure to embedded DU, there is a need to further investigate long-term effects in light of the high levels accumulated in some body tissues.

Introduction

Natural uranium consists of three isotopes: ^{238}U (99.276%), ^{235}U (0.718%), and ^{234}U (0.0056%). During the uranium enrichment process two products are produced, enriched uranium and depleted uranium (DU)—both contain different relative ratios of the three isotopes. Enriched uranium contains the higher amount of the fissionable isotope ^{235}U and is used for nuclear reactor fuel and nuclear weapons. DU has a lower ^{235}U content and is very dense. The DU used by the U.S. in kinetic energy penetrators is alloyed with titanium (0.75% by weight) to retard oxidation. This DU alloy is of concern because the U.S. military currently uses this metal for munitions and armament. During Operation Desert Storm a number of U.S. military personnel were wounded by shrapnel fragments consisting of DU [6, 7]. Since surgical removal can produce excessive tissue damage, these DU fragments were treated as conventional shrapnel and left in place in the wounded soldiers. The radiographs of injured soldiers show multiple embedded fragments ranging in size from 1 mm to over 5 mm in diameter. Uranium bioassays taken over 1 year after injury indicate that uranium was present in the urine well in excess of natural background uranium, up to 30 μg U/l of urine. DU fragments present a radiologically and toxicologically unique condition with unknown health risks. Congress has mandated the study of these risks.

This study evaluated the consequences of exposure to DU fragments in the rat model at the 1-day, 30-day, and 6-month time points of an 18-month study. Using an interdisciplinary approach, we assessed neurotoxicity, nephrotoxicity, and the histopathology of the tissue surrounding the fragment. Pathology including evaluation of neoplastic changes in several body tissues was also assessed. At the study's completion (18-month time point) we will develop a biokinetic model that describes the distribution of uranium from embedded fragments as a function of time.

Uranium Toxicity

Although the toxicity of embedded DU is unknown, numerous studies have addressed the consequences of inhalation, ingestion, and parenteral administration of other forms of uranium [27, 38, 45, 62]. After uranium is absorbed it circulates in the blood as the uranyl ion forming uranium-carbonate and uranium-albumin complexes [8, 26, 31]. As the uranium-carbonate complex passes through the kidney, it is filtered rapidly at the glomerulus where 60%–80% of absorbed uranium is excreted in the first 24 hours after acute exposure. The uranium not excreted is reabsorbed by the proximal tubules where it produces acute toxic effects. Uranium also enters the bone where it competes with calcium to form complexes with phosphate ions, thus becoming part of the bone matrix [3, 10, 16, 42]. This bone matrix then serves as a storage site from which uranium is slowly released back into circulation [23, 61]. The liver, muscle, and kidney are other major sites of uranium deposition, with a possible long-term storage mechanism in the kidney [19, 23, 27, 51, 62]. At low doses uranium may not readily distribute to the central nervous system (CNS) [45]. However, with higher doses (8 mg/kg/day orally for 4 weeks) brain uranium levels are comparable to those in liver and in bone [45], major sites for uranium accumulation.

Acute morphological and biochemical changes of the kidney result from uranium exposure [8, 26, 31, 42]. The glomerular epithelial architecture is altered [25] and cellular necrosis occurs in the proximal tubules near the corticomedullary junction in the kidney [2, 17, 18]. In addition, polyuria, enzymuria, glucosuria, and increased excretion of amino acids result [8, 9, 26, 63]. Exposure to high doses of either soluble or insoluble forms of uranium can lead to acute renal failure and death [43, 57]. Environmental stressors such as restricted diets or changes in housing conditions significantly enhance uranium toxicity [1, 4].

Few studies have addressed the chronic toxicity of uranium; and the results available are conflicting. Galibin and colleagues [14] reported severe renal toxicity in rats that inhaled the slightly soluble uranium compound, ammonium diuranate (1 or 8 mg/m^3) for 128 days. Urine protein and blood, non-protein nitrogen, were elevated. Sloughed dead

cells and abnormal regenerating cells were seen in the proximal tubules. These animals recovered, although the total number of tubules was reduced with an accompanying increased proportion of connective tissue in the kidney. In contrast, Leach et al. [29, 30], found no renal toxicity in rats repeatedly exposed for a period of 12 months to uranium dioxide dust (5 mg/m^3) (or in dogs or monkeys exposed for 5 years). Yet uranium concentrations in the kidney were as high as 1.1 μg U/g kidney wet weight in the rat (8.3 in the dog and 17.0 in the monkey)—levels reported to cause acute renal toxicity [23]. Thus, the chronic effects of uranium exposure remain for the most part unresolved [8].

The threshold concentration of kidney uranium levels in humans that results in kidney chemical toxicity is in dispute [8, 26, 52]. While the Nuclear Regulatory Commission has set the level at 3 μg/g kidney for renal damage in humans, there is evidence from both human and animal reports that this level could be much lower. For example, chronically exposed uranium mill workers, whose kidney uranium levels probably did not exceed 1μg U/g [54], showed mild renal dysfunction with increased urinary excretion of $beta_2$-microglobulin and various amino acids. In rats exposed subchronically to low doses (cumulative dose: 0.66 or 1.32 mg/kg) of uranyl fluoride, kidney uranium levels as low as 0.7 to 1.4 μg U/g, wet kidney, produced cellular and tubular necrosis of the proximal tubule, proteinuria, and enzymuria [9]. These changes in rat renal function, however, were temporary, with complete recovery within 35 days after exposure. These studies are important because they indicate that renal injury can occur at kidney uranium levels well below the 3.0 μg U/g limit.

Neurological effects have been reported with uranium exposure. In uranium workers excreting up to 200 μg U/l in their urine, normal mental function was disrupted [24]. One case study linked the handling of a uranium bar and a subsequent increase in stool uranium with foot cramps, leg pain, and abnormal gait [15]. With oral and subcutaneous administration of relatively high doses of uranyl acetate (210 mg/kg and 10 mg/kg, respectively) rats exhibited tremors [11]. The uranyl ion has been demonstrated to enhance muscle contraction with acute local concentrations of 200–400 μM [13, 32]. At the neuromuscular junction in the mouse, multiple sites of action were identified, including increased duration of the muscle action potential, broadening of the compound nerve action potential, increased amplitude and quantal content of the endplate potential, and increased frequency of the miniature endplate potentials [32]. These studies indicate that embedded DU fragments could lead to neural damage, affecting both motor and cognitive function. The CNS effects of uranium toxicity can result from secondary mechanisms since hormonal changes, electrolyte disruption, and immune responses can all influence nervous system activity [47].

Local-Tissue Response and Capsule Formation

Foreign bodies in tissue elicit an immune response that can result in encapsulation. Even when encapsulated, DU fragments provide a local, chronic source of alpha-radiation. Within 10–15 cells of the fragment, the dose rate is expected to be approximately 8.5 Gy/yr. This radiation could result in injury or damage to local muscle or nerve tissue (axonal injury, demyelination) [48, 58]. In addition, capsule formation around a DU fragment in close proximity to a nerve could increase the risk of nerve-compression injury.

Encapsulation could limit the chemical toxicity of DU fragments by decreasing the rate of release of metal, as has been observed with lead [35]. Encapsulation can result in the formation of pseudocysts. Pseudocysts have been observed that contained fluid with very high concentrations of soluble lead and insoluble lead dioxide particles [33, 35] with "black pigment...firmly adherent..." to portions of the inner wall of the capsule [33]. If these cysts should rupture, the rapid release of this fluid could cause period spikes in circulating lead levels and result in acute lead toxicity 5 to 40 years after the initial injury [33, 35, 59]. Similar type lesions may form around DU fragments. Intracapsular fluid may contain high concentrations of both soluble and insoluble DU. Tonry [55] demonstrated that DU disks formed both a soluble fraction and black insoluble particulates when emersed in simulated lung fluid. After a large fragment (approx. 20 mm)

was removed from a U.S. soldier 17 months after he was wounded, the surgeon [28] noted that the fragment was encased in a fibrous capsule. When the capsule was breached, approximately 1–2 ml of a black fluid "gushed forth" from the cystic space.

DU can cause both local and systemic toxicity through a variety of mechanisms. This study characterizes many of the potential sites of pathology that can result from long-term exposure to DU fragments and will provide a rationale for treatment of military casulties. A dose-ranging study determined the number of DU pellets required to obtain uranium levels in the range of 0.7 to 1.4 μg/g wet weight of kidney [3]. This level of uranium was reported to produce early signs of renal damage as measured by both biochemical and histopathological changes [9] and defined the high dose in the present toxicological study. The low dose was chosen to produce no measurable acute toxicity; and the medium dose was selected to produce moderate toxicity. This experiment uses these three doses to evaluate neurotoxicity, nephrotoxicity, and histopathology and to determine uranium distribution for biokinetic modeling.

Neurotoxicity was assessed by (a) a battery of behavioral tests to assess functional consequences, and (b) conduction velocity studies in motor nerves to uncover any peripheral neuropathies. Behavioral tests have frequently been employed to detect and characterize potential neurotoxic effects in rodents and have been used extensively in animal toxicity studies [44]. The neurobehavioral battery consisted of (i) a functional observational battery (FOB)—a series of tests designed to assess the neuromuscular, autonomic, and sensory integrity of the rat [12, 36, 37, 39, 40], (ii) an automated test of locomotor activity, and (iii) the passive avoidance test used to evaluate memory. Electrophysiological experiments monitored nerve conduction velocity and integrity of the neuromuscular response. Nerve conduction velocity studies have been used clinically for many years to diagnose peripheral neuropathies and can even detect subclinical neuropathy induced by lead exposure [20, 41, 49].

Markers of renal function in the urine and plasma were used to assess nephrotoxicity. Altered creatinine clearance and proteinuria can indicate glomerular damage, although tubular changes can also contribute. Increased urine content of enzymes such as lactate dehydrogenase (LDH) and N-acetyl-β-glucosaminidase (NAG) have been interpreted to reflect tubular damage [46]. In addition, the appearance of glucose in the urine can indicate alterations in tubule reabsorption. These markers have demonstrated sensitivity with acute uranium nephrotoxicity [8, 9, 31, 63] and should indicate any toxicity that might result from long-term exposure to DU fragments.

Capsule formation and the sporadic release of pseudocyst-fluid contents can significantly influence the time, course, and concentration of uranium distributed throughout the body. The encapsulation process and pseudocyst formation was characterized at the time of euthanasia (1 and 6 months after implantation); surrounding tissues are histologically examined and any capsular fluid was analyzed for its uranium content. In addition, tissues that are known to accumulate soluble uranium or uranium particulates (liver, bone, kidney, spleen) [19, 27, 29, 30, 61, 62] were histologically evaluated.

Although the distribution of uranium in the rat has been characterized for a variety of routes of internalization (inhalation, ingestion, and parenteral administration of soluble compounds), this information is not available for embedded fragments. We measured uranium in urine, plasma, kidney, bone (tibia and skull), liver, spleen, brain, and skeletal muscle that is proximal and distal from the embedded pellets. Uranium is transported in plasma and urine and is stored in the kidneys and bone [19, 27, 61, 62]. Uranium has been detected in the liver and spleen of animals [19, 29, 30] as well as in human subjects [23]. The skeletal muscle is being sampled to determine the local concentrations of uranium. The brain was chosen because of the paucity of data and the need to assess whether any neurological effects observed were caused by the direct or indirect interaction of uranium in the body. These data will allow a rat biokinetic model for implanted DU fragments to be developed.

Methods

Approach

This report describes the uranium distribution data obtained after 1 day, 30 days and after 6 months in rats exposed to DU. Data from additional time points, 12 and 18 months, will be presented in future reports. Rats from the 30-day and 6-month time points were thoroughly evaluated for changes in behavior, peripheral nerve function, CNS excitability, renal function, and tissue histology, including capsule formation. In addition, data on tissue uranium levels from a subgroup of rats will be used (following the 18-month time point) to develop a biokinetic model to predict uranium distribution.

Rats were randomly assigned to five treatment groups: 1) a non-implanted sham-surgical control group, 2) rats implanted with tantalum (Ta) to control for fragment implantation, 3) rats implanted with low-dose DU, 4) rats implanted with medium-dose DU, and 5) rats implanted with high-dose DU. In the low-dose and medium-dose groups, Ta was substituted for a fraction of the DU pellets to keep the total number of implanted fragments constant. Half of the total number of pellets were implanted in each thigh.

Based on the variance of control data for neurological effects, a group size of 15 rats was determined to be necessary to observe significant changes of 20% or greater at the $p<0.05$ level. Additional animals (20 rather than 15) will be implanted for the 18-month time point with the expectation of approximately 25% natural mortality [64–66]. Five of the rats in each experimental group provided tissue for uranium quantification.The remainder will be evaluated for histopathology. Two-way analysis of variance was used to test statistical significance of any changes. Newman-Keuls test was used for multiple comparisons. In all analyses, statistical significance was accepted at the $p<0.05$ level.

Subjects

Sprague-Dawley rats (8–10 weeks of age) were maintained in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals* (NIH Publication No. 86-23). Upon arrival, rats were quarantined and screened for diseases. Except during urine collection, all animals were housed in plastic Microisolator rat cages with hardwood chips as bedding. Commercial rodent chow and water were provided *ad libitum*. Rats were on a 12-hr light/dark cycle. Separate groups of rats were used for each time point.

Fragments

DU fragments consisting of 99.25% DU and 0.75% titanium by weight were obtained from Oak Ridge National Laboratories, Oak Ridge, TN. The uranium isotopes present are ^{238}U (99.75%), ^{235}U (0.20%) and trace levels of ^{234}U. This is the same DU alloy used in U.S. military munitions. Tantalum (Ta) fragments were obtained from Alfa Products, Ward Hill, MA. Ta was chosen as the control substance because it is a biologically inert metal [22] with a similar mass to uranium and is frequently used in human prostheses [21, 53]. Each fragment (both DU and Ta) was approximately 1 mm diameter x 2 mm long.

Experimental Groups

Rats were divided into five experimental groups: 1) nonsurgical controls, 2) Ta controls, 3) DU low dose, 4) DU medium dose, and 5) DU high dose. The preliminary dose ranging study determined that 20 DU pellets produced urine uranium levels of 262 ± 99 μg U/l and kidney levels of $1.22 \pm .31$ μg/g after 2 weeks of exposure. This dose was chosen as the high dose because it was well tolerated by the rats but was expected to produce kidney toxicity. Consequently, all surgically implanted animals have a total of 20 pellets of either Ta, DU or a mixture. Low-dose DU rats were implanted with four DU pellets and 16 Ta pellets. Medium-dose DU rats were implanted with 10 DU and 10 Ta pellets.

Surgery

The DU and Ta pellets were cleaned and chemically sterilized prior to implantation. The pellets were immersed in industrial detergent, rinsed in absolute alcohol, soaked in a 50% nitric-acid solution for 3 min and then rinsed with water. This procedure completely removed the oxide formation on the surface of the DU pellet [55].

Anesthesia was induced with ketamine hydrochloride (80 mg/kg) in combination with xylazine hydrochloride (4 mg/kg) given i.p. Fragments were implanted within the gastrocnemius muscle, spaced approximately 8–10 mm apart on the lateral side of each leg. Prior to surgery the surgical sites were shaved and cleansed with betadine, a topical disinfectant. Scalpel incisions were made through the skin; and pellets were inserted into the muscle with a 16-gauge needle with plunger. Incisions were closed with absorbable sutures and surgical cement. Rats were closely monitored following surgery until they were ambulatory; and an analgesic (Demerol, 10 mg/kg, i.m.) was administered if needed. A veterinarian regularly examined the surgical sites for signs of inflammation, infection, and local DU toxicity.

Behavioral Neurotoxicity

The functional observational battery (FOB) consisted of behavioral evaluations (home-cage, handling, and manipulative) and several physiological measures. The recorded parameters are listed below and are grouped according to the following functional domains: 1) autonomic: lacrimation, salivation, palpebral closure, piloerection, defecation, urination; 2) sensorimotor reactivity: tail pinch response, tactile response, click response, approach response; 3) neuromuscular: gait, foot splay, forelimb and hindlimb grip strength, righting reflex; and 4) CNS excitability: arousal, posture, ease of removal from cage, handling reactivity, convulsions, and locomotor activity.

The observer was blind as to the identity of each group. The behavioral battery commenced with brief home cage observations during which time the observer described the posture, and the existence of tremors or convulsions, and palpebral closure. The rats were then removed from their cage and rated for ease of removing and handling. While handling the rat, presence of piloerection and the degree of lacrimation and salivation were observed. The animals were then placed in an open field with a perimeter barrier on clean absorbent white paper for 3 min. The number of rears, the gait, level of alertness, stereotypy (repetitive movements, e.g., head weaving), unusual behaviors (e.g., writhing), and the number of fecal boli and urine pools were recorded.

Sensorimotor responses also were determined and included: approach response to a blunt probe, touch on the rump (tactile response), click response (auditory response), and pinch on the tail using forceps. Next, neuromuscular responses were determined and included: righting reflex, forelimb and hindlimb grip strength using digital strain gauges [37], and landing foot splay [12]. The animals were weighed and their rectal temperature was determined using a digital thermometer. The FOB was conducted during the light portion of the light-dark cycle. Details of the FOB tests are in Moser et al. [40] and McDaniel and Moser [36].

Approximately 1 hr after the FOB, the rats were monitored for horizontal and vertical locomotor behavior. Motor activity was recorded for 1 hr using automated photocell activity cages (Digiscan Analyzer, Omnitech Electronics, Columbus, OH). On the day following the FOB and motor activity tests, animals were trained on a passive avoidance test. This test was used to determine whether DU affects memory function. The tests were conducted in a passive avoidance apparatus (San Diego Instruments, San Diego, CA) that consisted of two chambers (one lighted, one darkened) separated by a sliding door. The animal received a training trial during which time it was initially placed into the lighted chamber. The natural tendency was for the rat to enter the darkened chamber. When it did, it received a mild foot shock. During this acquisition phase, the rats were tested for eight trials or until criterion was met. The criterion was two consecutive trials during which the rat did not cross into the darkened chamber. Each trial was 3 min in duration with a 1 min intertrial interval. Seventy-two hours later

the rat was placed into the lighted chamber and retested. A comparison was made with the initial training session to see if memory of the task had been retained.

Conduction Velocities

One week following the behavioral testing, the rats were evaluated electrophysiologically. Rats were anesthetized with ketamine (80 mg/kg) with xylazine hydrochloride (4 mg/kg) i.m. (supplemented as necessary). The right sciatic nerve was exposed and bipolar stimulating electrodes were positioned along the nerve in the thigh close to the sciatic notch and in a second location close to the knee. A recording electrode was inserted into the medial gastrocnemius muscle to monitor the compound muscle action potential. Nerve temperature was monitored and maintained near 37° C with a heat lamp. The nerve was stimulated at a frequency of 0.2 Hz. Stimulus intensity was varied between approximately 10 and 100 V (0.1 ms duration) to determine the input-output relationship and the supramaximal stimulation parameters to use. Five muscle responses were averaged and the latency, duration, and amplitude of the potentials were measured. Conduction velocities were calculated by dividing the distance between the stimulating electrodes by the average latency difference between the time of onset of the compound muscle action potentials.

Duration of the muscle action potential reflects the synchrony of discharge. In general, the distal stimulating electrode will produce a faster, larger response than the proximal electrode. Greater dispersion and greater decrease in amplitude than normal would suggest nerve damage. For example, demyelinating disorders cause dispersion of the muscle action potential by slowing the nerve-conduction velocities [5, 50]. If dispersion occurs over a short segment, compression neuropathy may be indicated [5].

All stimulation and recording were controlled by a 486 PC using standard electrophysiological software (Axon Instruments). Data were analyzed with routines written in AxoBasic (Axon Instruments) and statistical analysis was done with RS/1 (BBN Software Products) routines. Two-way analysis of variance (for time and dose) was used to compare differences among the experimental groups.

Hippocampal-Slice Electrophysiology

At the termination of the conduction velocity experiment, the rat was euthanized by decapitation. The brain was quickly removed from the skull and was submerged in iced oxygenated artificial cerebrospinal fluid (ACSF). Both hippocampi were dissected out and sliced on a McIlwain tissue chopper (425 μm thick). Tissue was incubated at room temperature in oxygenated ACSF (see below) for 1 hr to allow recovery from the slicing procedure. During this interval, tissues were isolated from the rats for analysis of histopathology and DU content.

A single slice of rat hippocampus was then placed in a submerged slice chamber and perfused at a rate of 1–2 ml/min with warmed (30°C) oxygenated ACSF. ACSF has the following composition (in mM) 124 NaCl, 3 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 1.24 KH_2PO_4, 10 glucose, 26 $NaHCO_3$, pH 7.4, equilibrated with 95% O_2/5% CO_2. Extracellular recordings were obtained with glass microelectrodes filled with 2 M NaCl placed in s. radiatum and s. pyramidale of field CA1 to record the population synaptic potential (pPSP) and the population spike (PS), respectively. A stainless steel, concentric, bipolar stimulating electrode was positioned in s. radiatum of field CA1 to activate afferents. Constant current stimuli (0.1–1.5 mA, 300 μsec) were applied at a frequency of 0.2 Hz. Except when generating input/output (I/O) curves, the stimulus current was held constant at an amplitude that elicited approximately 30% maximal response.

To obtain I/O curves, stimulus intensity was varied from approximately 0.1 to 1.5 mA in 13 steps. Three responses at each current step were recorded and averaged. I/O curves were generated following a 30-min equilibration period. The three I/O curves (stimulus vs. PS, stimulus vs. pPSP, pPSP vs. PS) were analyzed with the data analysis software RS1 (BBN Software Products, Cambridge, MA). The responses at each stimulus intensity were averaged for all experiments at each time point. A sigmoid curve

was computer fitted to the points. Differences between curves were tested for significance by comparing the residual sum of squares for the curve fit to the data of each experimental condition with the residual sum of squares for the curve fit to all the data. Significance was accepted at $p < 0.05$.

Sample Collection

Following behavioral testing, blood and urine samples were obtained from all rats for analysis of renal function. To safely collect the blood samples, rats were immobilized by placing them in a Plexiglas restrainer. During each collection, 0.3–0.5 ml of blood was obtained from the tail vein using a 22-gauge needle. The blood was then centrifuged for 5 min at 3,000 x g. The serum was analyzed for uranium levels and/or for biochemical indices of renal function. Serum was stored at -70°C until ready for analysis.

Urine samples were collected by housing the rats in individual metabolism cages (23.5 cm diameter X 12 cm high) where they had continuous access to food and water. However, since these housing procedures have been shown to induce stress and thus increase the toxicity of uranium [4], the rats were acclimated to the metabolic cages for 5 days before the study began. The metabolic cages were disinfected and decontaminated between each animal use. A 24-hr urine collection sample was obtained from each rat and the volume recorded (10–20 ml). Urine collection at 4°C was unnecessary since enzyme activity was shown to be stable at room temperature for up to 24 hours [63]. After collection, urine was filtered to remove any debris and stored in plastic containers at 4°C until analyzed (less than 1 week).

Evaluation of Renal Function

Measurement of urine volume and osmolarity, urine levels of NAG, LDH, glucose, total protein, creatinine, and blood levels of glucose, urea, and creatinine were used as indicators of renal function. In addition, since weight loss may be indicative of nephrotoxicity, all the rats were weighed weekly throughout the study. Osmolarity of the urine was measured with a vapor pressure osmometer (Model 5100B, Wescor, Inc.). A Kodak Ektachem 700 Analyzer was used to determine plasma and urine levels of creatinine, glucose, and urea. Total urine protein was measured with a dye-binding assay (Coomassie Blue, BioRad) sensitive down to 1 μg. The activity of NAG was measured by the methods of Tucker et al. [56] using 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide as the fluorescent substrate (excitation wavelength = 356 nm; emission wavelength = 446 nm). The dilution of the urine for this assay eliminated the effects of any inhibitors present [56]. For LDH measurements, 1 ml of urine was dialyzed for 4 hr at 4°C with 1 liter of deionized water. LDH was quantitated with a colorimetric assay that measured a reaction product which was proportionate to LDH activity (Oxford Biomedical Research, Inc). Only 50–100 μl of fluid (urine or plasma) were required for each of these assays.

Although urine volume and osmolarity can vary greatly with fluid intake, these measures provide physical indicators of renal function. For example, acute kidney failure drastically decreases urine volume; while moderate renal toxicity can increase urine output, as is seen with uranium exposure [11]. Osmolarity can reflect the ability of the kidney to concentrate (or dilute) the urine. Plasma urea also changes with renal insufficiency. Since the rate of urea formation is proportionate to the rate of protein metabolism, other factors such as hepatic injury or altered protein intake can affect the measured urea in plasma. A small concentration of protein is normally present in the urine. Increases in total urine protein could result either from glomerular leakage or failure of tubule reabsorption. Urinary enzymes are sensitive, non-invasive markers of toxicity primarily in the kidney tubules [46]. NAG is a lysosomal enzyme found in proximal renal tubule cells. LDH is a cytosolic enzyme of the tubular epithelium.

Creatinine clearance is a commonly used measure of glomerular filtration rate in the rat, despite a significant but constant tubular secretion. The use of an intrinsic metabolite has an obvious advantage over inulin or mannitol which (although not secreted) must be infused. Interpretation must be cautious since tubular injury with uranium could cause an underestimation of the glomerular filtration rate regardless of the marker used [8]. Creatinine clearance

(C_c) was calculated from the equation: $C_c=U_c*V_u/P_c$ where U_c and P_c are the creatinine concentrations in urine and plasma, respectively; and V_u is the rate of urine production (ml/min).

The appearance of glucose in the urine occurs when the tubule reabsorption maximum from the filtrate is exceeded. This can occur with hyperglycemia or with a decrease in tubular reabsorption capacity. Measuring both urine and plasma glucose helps to distinguish between these two possibilities. Changes in reabsorption is reflected in the calculated fractional excretion (FE): $FE = (U_g/P_g))(U_c/P_c)$; where U_g and P_g are the glucose concentrations in urine and plasma, respectively.

The assays mentioned above provide a broad spectrum of measures of kidney toxicity. Many of these indicators have been shown to be very sensitive in acute uranium toxicity [8, 31]. Increased urine glucose, without concurrent increases in plasma glucose, was one of the most sensitive indicators [8, 9]. LDH and to a lesser extent NAG increase following uranium exposure [8, 31]. A transient increase in urine volume and the appearance of protein in the urine also occurred with acute uranium toxicity [31]. These measures were used together as indicators of kidney toxicity and were carefully interpreted and correlated with histopathology. Two-way ANOVA was used to test the statistical significance of any changes.

Histopathology

Immediately following euthanasia on the day of electrophysiological analysis, tissue samples from bone (tibia, skull), hippocampus, sciatic nerve, kidney, liver, spleen, and fragment capsule with associated skeletal muscle were obtained for histological examination or uranium measurement. Based on the literature, these are the most likely tissues to show increased levels of uranium [19, 27, 29, 30, 61, 62]. In the preparation of the samples, standard procedures for handling biologic specimens were used. Tissues were perfused, embedded, mounted, and stained with hematoxylin and eosin stain (H & E) [34]. Specialized stains were used to demonstrate specific lesions or to further delineate lesions not well defined by the H & E stain. For example, silver stains were used on neural tissue to delineate nerve fiber disruption or degeneration [34].

The pathologist evaluating the tissue was blind to the experimental group from which the tissue was obtained. The pathologist generated a 0 to 4 score to evaluate the degree of microscopic changes observed, where 0 = no change, 1 = minimal change, 2 = mild change, 3 = moderate change, and 4 = marked or severe change. All tissue changes observed in the rats implanted with DU were contrasted and compared to the identical tissues taken from the controls. If there were significant changes noted in a particular system (e.g., the renal system), a detailed statement of criteria for the 0 to 4 score was stated by the pathologist at the time of interpretation.

Uranium Measurement

Tissue samples were frozen and shipped by overnight courier on dry ice to Battelle, Pacific Northwest Laboratories, for analysis of uranium content. The samples were stored at -70° C until the wet ashing procedure. Wet ashing involves 12 cycles (over 3 days) of treating samples with 2 ml of 16 N nitric acid followed by several hours of heating, brief cooling, the the addition of 0.5 ml of 30% hydrogen peroxide, and reduction of the volume to approximately 0.5 ml. After this, samples were heated to dryness, dissolved in 2 ml of 4 M nitric acid with warming and were then filtered through 0.45 μm syringe filter units. For analysis, 0.5 ml of sample or identically handled standards were dissolved in 2 ml of Uraplex reagent. The samples were analyzed with a Kinetic Phosphorescence Analyzer (KPA-11, Chemchek Instruments Inc., Richland, WA). Background measurements were made using 4 M nitric acid. Calibration curves were established prior to sample analysis. Measurements included analysis of relative standard deviations and correlation coefficients of the luminescence decay curve.

Results

Because of the staggered experimental schedule required for complete analysis of the numerous experimental endpoints, all subjects have not been evaluated to date. Sample sizes, therefore, will vary.

Uranium Distribution

Tissues and fluids from five rats from each of the five experimental groups for the 1-day, 30-day, and 6-month time points have been analyzed for uranium content by Battelle Pacific Northwest Laboratories. Urine was not collected from the rats at the 1-day time point because of complications introduced by the surgery and the procedures for the collection of the fluid. At all time points uranium distributed primarily to bone and kidney in a dose-dependent manner (figures 1, 2, 3). Even at day 1, uranium was present in these tissues (figure 1). By day 30, the levels in kidney and bone increased; and uranium was evident in the urine (figure 2). In addition, the high-dose DU animals showed evidence of uranium distributing to the spleen and to the brain. At 6 months, the uranium levels in tibia, kidney, urine, and brain had continued to rise; while levels in the skull had plateaued (figures 3–9). Distribution to the spleen in the high-dose DU animals leveled out, but for the animals with low and medium doses, it continued to rise (figure 8). Uranium levels in the liver were comparable to controls at the 30-day time point but increased slightly at 6 months (data not shown). Muscle levels were in general quite variable. Some of the muscle samples that were in close proximity to the DU pellets showed exceptionally high levels of uranium. It is our belief that these high levels resulted

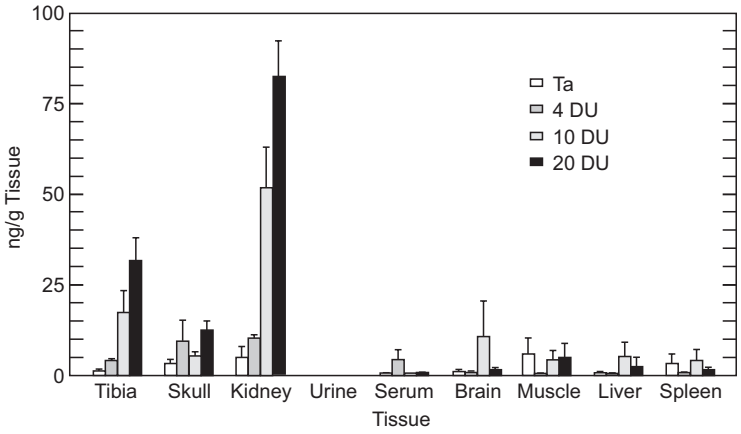

Fig. 1. Uranium distribution in rat tissue 1 day post DU-pellet implantation.

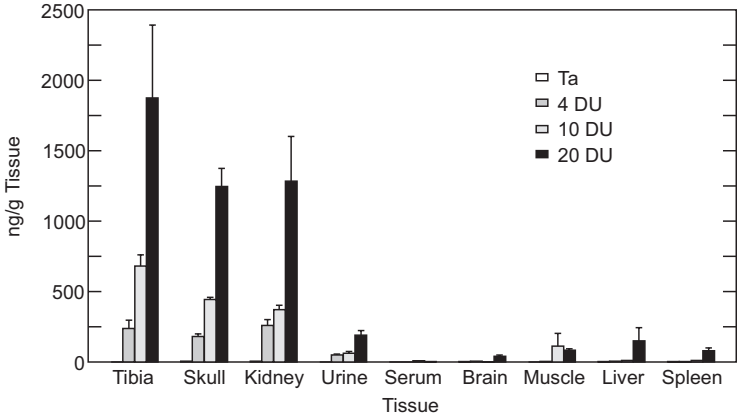

Fig. 2. Uranium distribution in rat tissue 30 days post DU-pellet implantation.

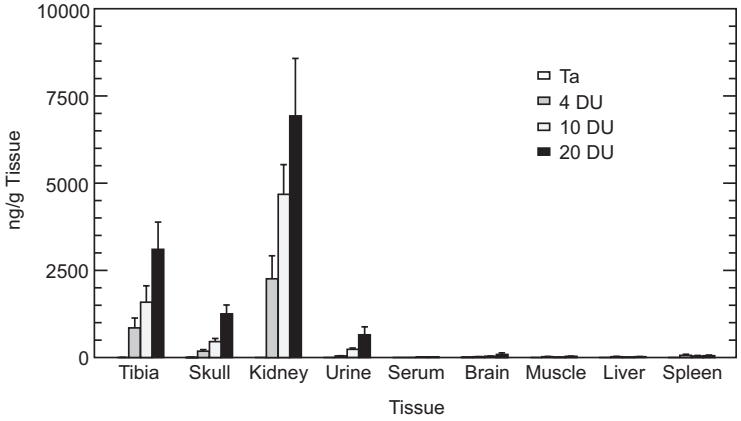

Fig. 3. Uranium distribution in rat tissue 6 months post DU-pellet implantation.

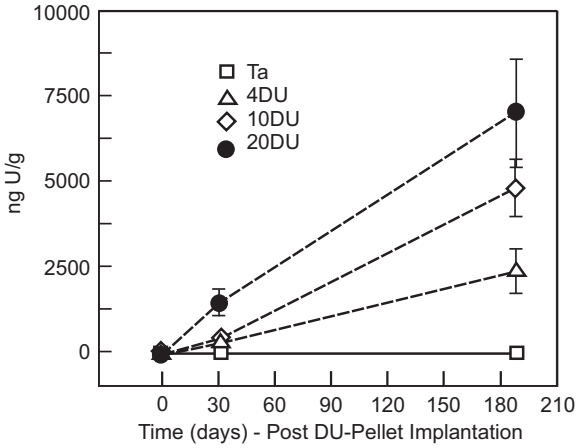

Fig. 4. Uranium distribution in kidney.

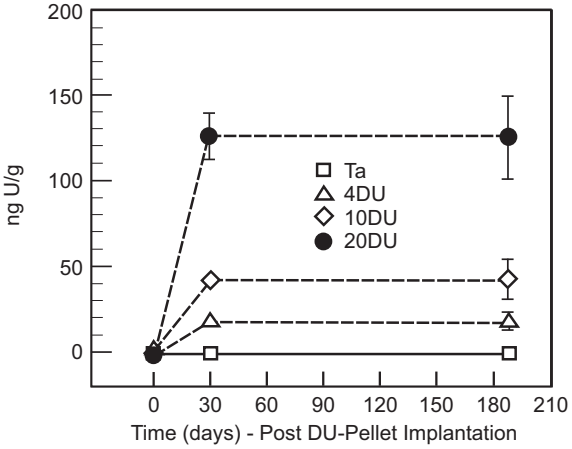

Fig. 7. Uranium distribution in skull.

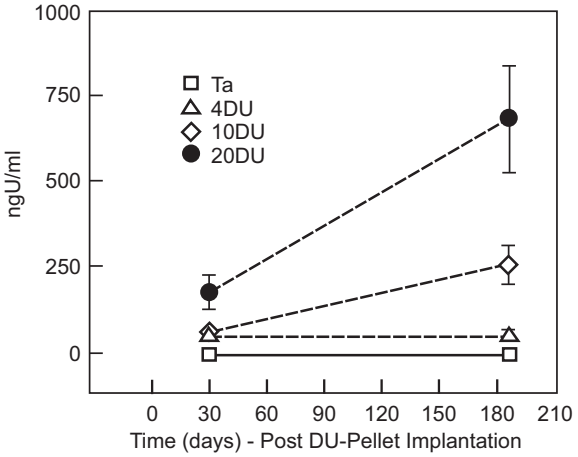

Fig. 5. Uranium excretion in urine.

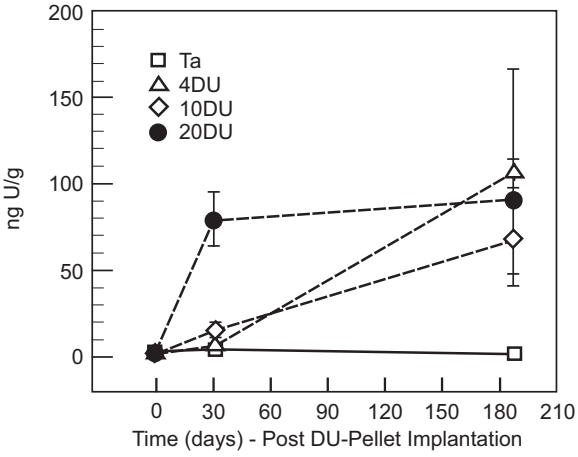

Fig. 8. Uranium distribution in spleen.

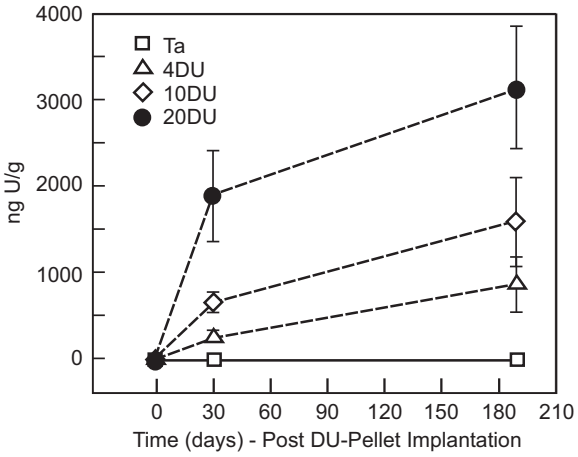

Fig. 6. Uranium distribution in tibia.

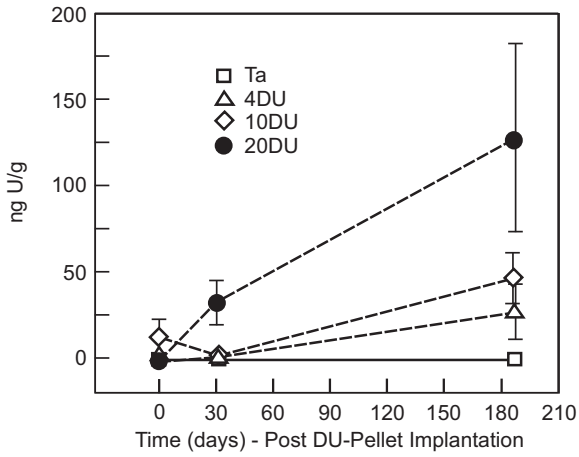

Fig. 9. Uranium distribution in brain.

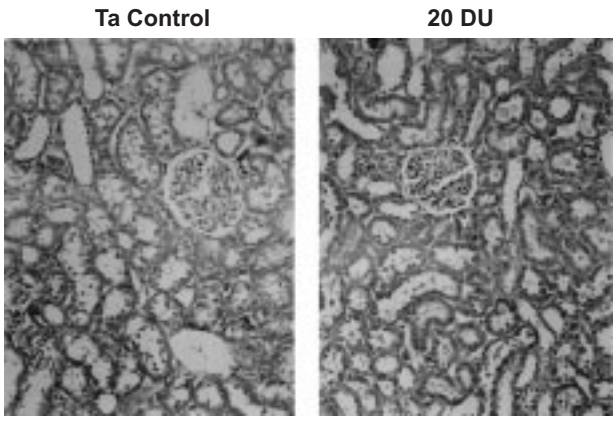

Fig. 10. Renal cortex (50X) at the 6-month time point in one high-dose DU rat and in a Ta control.

directly from fragments of the implanted pellets in the analyzed samples. This "contamination" could have occurred during the removal of the pellets at time of necropsy or might have happened by flaking and redistribution *in vivo*. Further analyses are expected to clarify this issue.

Histopathology

Tissues from (bone (tibia and skull), kidney, spleen, liver, brain, and muscle (proximal and distal)) were excised and fixed for histopathological analysis. No histological evidence of toxicity was observed. Figure 10 shows the renal cortex at 6 months in one high-dose DU rat and in a Ta control. There is no evidence of cellular necrosis, inflammation, or fibrosis. During excision of the pellets it was observed that the DU pellets but not the Ta pellets were associated with adherent tissue. Even at 6-months, a capsule had not fully formed around the DU pellets and dark fluids were not observed near the fragments.

Nephrotoxicity

The urine and serum samples were analyzed for biochemical markers of kidney toxicity. Osmolarity, 24-hour volume, pH, and urine levels of glucose, protein, NAG, LDH, urea nitrogen, serum glucose, and serum urea nitrogen were not significantly altered at either the 30-day or 6-month time points. The 6-month data for urine osmolarity, glucose, protein, and NAG are shown in figures 11-14. Creatinine

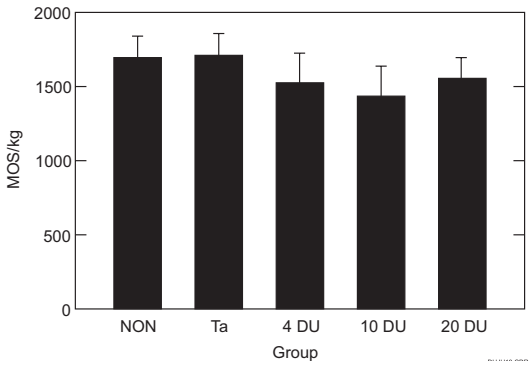

Fig. 11. Osmolarity of urine at the 6-month time point.

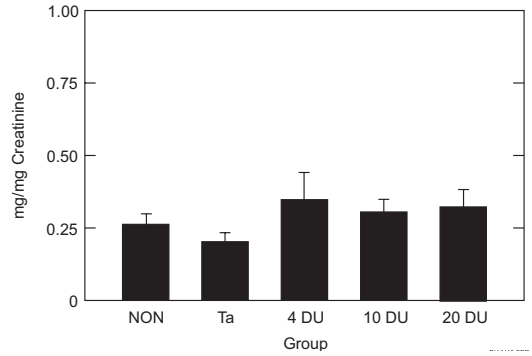

Fig. 12. Urine glucose at the 6-month time point.

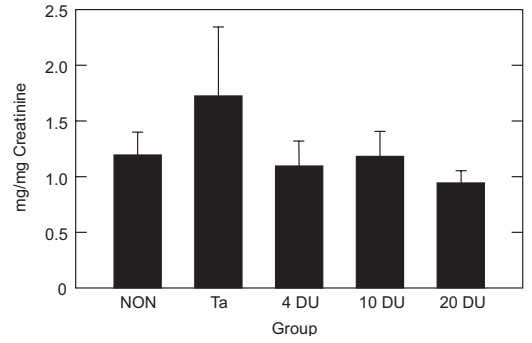

Fig. 13. Urine protein at the 6-month time point.

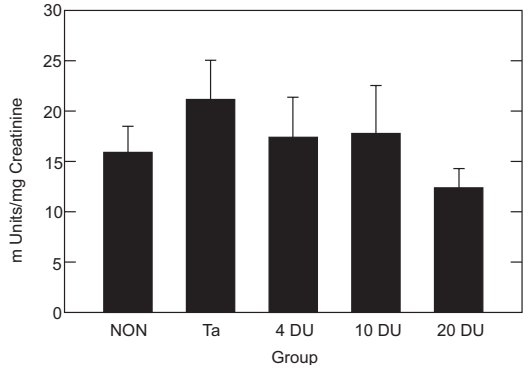

Fig. 14. Urine NAG activity at the 6-month time point (N = 9–11/group).

clearance was not significantly different among the experimental groups (figure 15). Fractional excretion (FE) of glucose (glucose clearance/creatinine clearance) was similarly not significantly affected by the experimental procedures—with all groups showing an FE between 0.0025 and 0.0038 at the 30-day time point, and between 0.0008 and 0.0015 at the 6-month time point. The differences were not statistically significant ($p>0.2$).

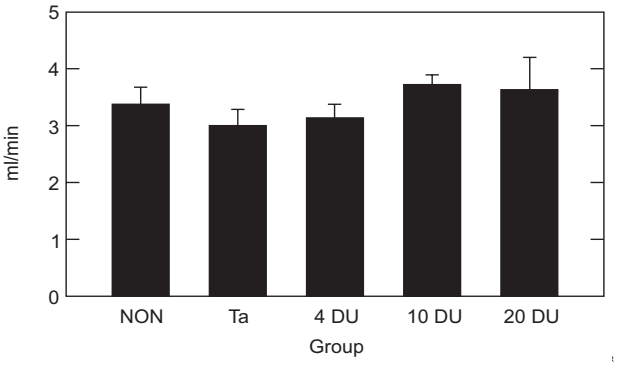

Fig. 15. Creatinine clearance at the 6-month time point.

Neurotoxicity

Animals were evaluated for body weight and for changes in the functional observation battery (FOB), locomotor activity, and passive-avoidance learning. The rats were weighed weekly. All steadily gained weight. At a number of time points, animals in the high-DU dose group have shown significant differences in body weight compared to Ta controls (figure 16) ($p<0.05$). Although not statistically significant, the medium-dose DU animals show the same trend toward lower body weight compared to the control animals.

The FOB did not reveal any significant differences among the experimental groups. No significant differences were observed in body temperature at 30 days or at 6 months. Sensorimotor, neuromotor, and autonomic responses as well as locomotor activity showed no significant differences across the experimental groups at either time point. At the 6-month time point, in all groups, the initial locomotor activity was high when the animals were first placed in the activity monitors because of exploratory behavior which subsided over time (figure 17). Grip strength of the hindlimbs and forelimbs was not significantly altered by DU exposure (figure 18). Conduction velocity measurements from the nerves of the hindlimb also did not reveal any differences among the experimental groups at either time point. Data for the 6-month time point are illustrated in figure 19.

Passive avoidance was used as a measure of learning ability. Any rat that failed to cross to the darkened compartment was dropped from the study.

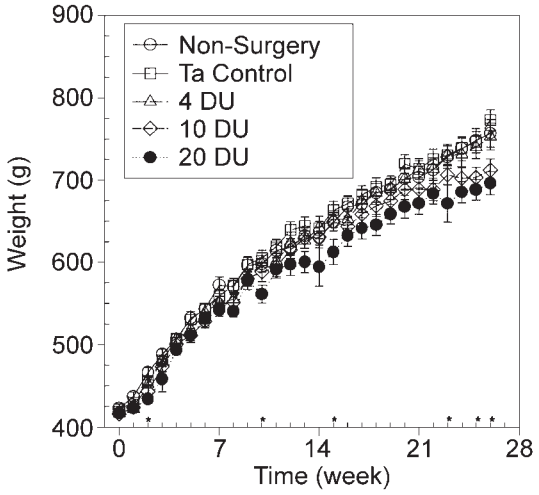

Fig. 16. Body weight. *20 DU differs from Ta control ($p<0.05$).

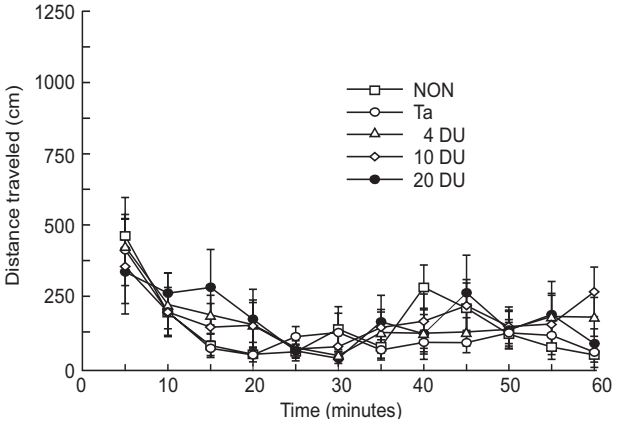

Fig. 17. Locomotor activity at 6 months post DU-pellet implantation (N = 12–13/group).

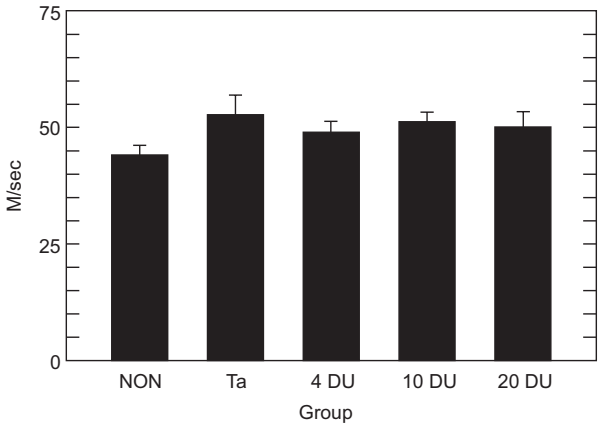

Fig. 19. Conduction velocity at the 6-month time point (N = 9–10/group)—sciatic nerve.

As a consequence, the number of subjects reported for this endpoint is less than the number of animals in the study. For both the 30-day and the 6-month time points, there were no significant differences among the five experimental groups for performance on the passive avoidance test (N = 7–10/group at 30 days; N = 4–9/group at 6 months). All animals learned to avoid the mild foot shock within 2–3 trials.

The latency to initial crossover was also not significantly different among groups (figure 20).

At the 30-day evaluation only the high-dose DU-implanted rats had distribution of uranium to the brain; therefore, hippocampal electrophysiology was assessed only in this group and in the the Ta controls at the 6-month evaluation. The population

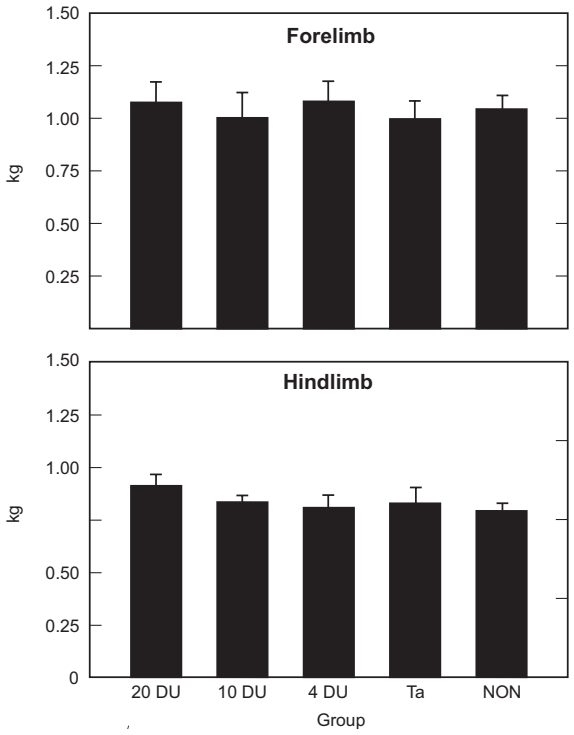

Fig. 18. Grip strength at the 6-month time point (N = 9–11/group).

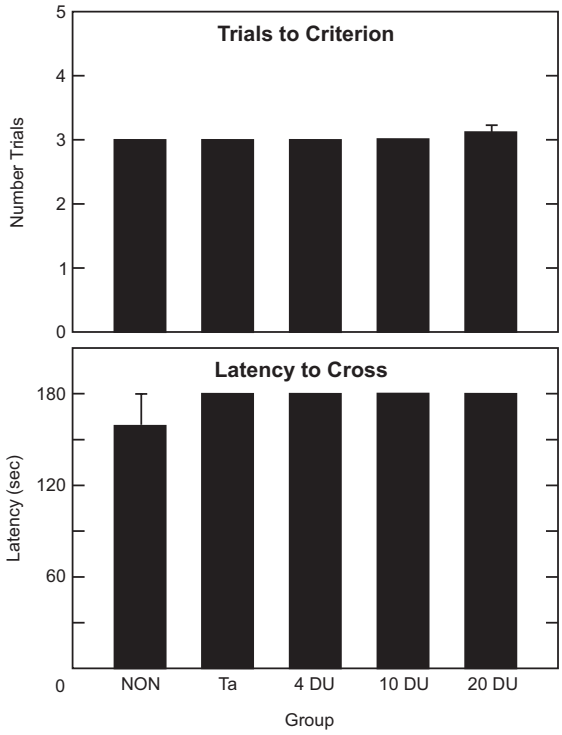

Fig. 20. Passive avoidance at the 6-month time point (N = 4–9/group).

spike in the hippocampus of the DU-implanted animals was significantly smaller than in the controls (figure 21). In contrast, the differences in the size of the synaptic potentials were not statistically significant (data not shown). The input-output relationship reflecting the ability of the synaptic potential to elicit the population spike indicated that in the high-dose DU animals this process was significantly impaired ($p<0.05$).

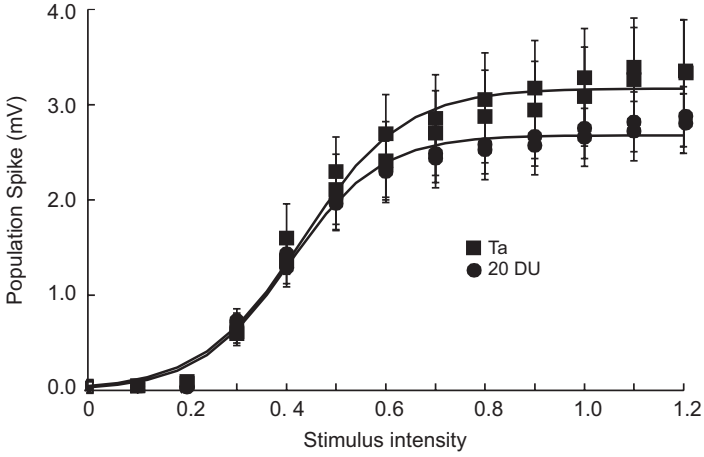

Fig. 21. Population spike is decreased in 20-DU pellet rats at 6 months.

Miscellaneous Observations

In the 6 months since pellet implantation, several pathological conditions have resulted in the removal of subjects from this study as recommended by our veterinary staff.

Five rats developed dental problems. Their upper teeth broke off and their lower teeth grew abnormally. All of these rats were implanted with DU pellets (one low dose, two medium dose, two high dose). These animals had difficulty eating, showed weight loss, and were euthanized.

Three rats (one low-DU dose, one medium-DU dose, one high-DU dose) were euthanized because they exhibited substantial weight loss accompanied by gasping or coughing. On necropsy they were found to have gas-filled intestines. Two animals (one Ta control, one medium-dose DU) were euthanized when they developed skin sores that failed to heal with antibiotic treatment.

Five additional animals died or were euthanized when observed moribund. In three of these (two low-DU dose, one Ta control) no abnormal pathology was observed. The fourth rat (non surgical control) had an enlarged liver; and lymphosarcoma was suspected. Necropsy of the fifth rat (nonsurgical control) revealed bladder stones.

Conclusions

The data presented here describe the distribution of uranium from intramuscularly embedded pellets to body tissues over 6 months and characterize the physiological and behavioral consequences of this exposure. The most striking findings of this study to date are 1) the absence of measurable nephrotoxicity despite very high levels of uranium in the kidney and urine, and 2) the distribution of uranium to the brain and the changes in neuronal excitability in the hippocampus.

Uranium levels in the kidneys of the experimental animals implanted with DU exceed the levels considered to be toxic in both animal models and humans. The medium-dose and high-dose groups averaged kidney levels greater than 3 μg/g, the level set by the Nuclear Regulatory Commission for renal damage. At 6 months, the high, medium, and low doses of DU pellets resulted in 6.9±1.7, 4.7±0.8, and 2.3±0.6 μg/g kidney, respectively. Examination of the time course of the uranium levels in the kidney suggests that these values may continue to rise. Urine levels of uranium in the high-dose and medium-dose groups (674±156 and 243±52 μg/l, respectively) exceed the levels found in Desert-Storm veterans (Keogh, personal communication) while the low-dose group (46±13 μg/l) showed levels comparable to that population. As with the kidney levels of uranium, the urine levels continue to rise over time. Despite these high levels, our data do not demonstrate any signs of nephrotoxicity. Chemical form, route of administration, and the dose of uranium exposure can all affect the toxicological consequences and distribution of uranium. It is possible that chronic exposure to uranium allows tolerance to higher concentrations of the metal. The studies of Leach et al. [29, 30] demonstrated an absence of renal toxicity in rats following chronic inhalation exposure to uranium dioxide that produced kidney uranium levels up to 1.1 μg/g. In contrast, Diamond et al. [9] observed acute, but reversible renal toxicity in rats at levels as low as 0.7 μg/g following i.v. injection of uranyl fluoride. The absence of effects in our present study does not preclude the possibility that toxicity will develop with longer exposures to uranium.

At the 30-day time point, uranium was observed to distribute to the brain in the high-DU dose animals. This was in agreement with the literature [45] in which uranium did not accumulate in the brain at the lower DU doses. At 6 months, however, substantial amounts of uranium are accumulating in the central nervous system. Although behavioral measures did not reveal any adverse effects from this uranium, an electrophysiological assessment of hippocampal neuronal activity demonstrated that excitability was impaired. It is possible that the electrophysiological changes are too subtle to produce a behavioral manifestation. Alternatively, it is possible that the behavioral tests we have performed are not sufficiently sensitive to reveal an existing cognitive deficit. It also needs to be emphasized that only the high-dose DU animals were assessed; and these animals had levels of uranium in their urine that significantly exceeded the levels found in any of the veterans. Future studies will determine the electrophysiological correlates in all of the dose groups. In addition, it will be important to assess the process of long-term potentiation, a physiological correlate of memory and learning in the brain slice preparation.

Bone, like kidney, is well accepted as a primary reservoir of uranium. As with kidney, uranium appears to be continuing to accumulate in the tibia of the DU-embedded rats. In contrast to the marrow bone, skull levels are high but after 30 days appear to have become saturated in their uranium concentration. This could be the difference between marrow and non-marrow containing bone. Perhaps more likely, the difference could be due to the continued growth of the tibia during this time period and the much slowed growth of the skull. If uranium is deposited with bone growth, we would predict that over the next 6 months (i.e., by the 1-year time point) levels in the tibia will also begin to saturate. Future studies should resolve this issue.

Other organs accumulate uranium to varying degrees. At 30 days, concentrations in the liver were not above background while concentrations in the spleen and muscle were significantly higher. At 6 months, levels in the liver had significantly increased

but were still exceeded by levels in the spleen. Muscle levels of uranium raise the possibility that neuromuscular deficits will develop through heavy-metal effects. Spleen levels may have immunological consequences. Future studies are planned to address these issues.

These data suggest the potential health hazards associated with exposure to depleted uranium shrapnel. The data indicate that chronic exposure to uranium may not be as toxic to the kidneys as had been anticipated from acute exposure studies. In contrast, the distribution of uranium to the brain and the observed electrophysiological changes could in time result in alterations in cognitive function. Since the use of depleted uranium armaments is expanding, it is increasingly important to be aware of health risks associated with exposure in order to formulate an appropriate protocol for handling casualties with DU shrapnel.

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13. ABSTRACT *(Maximum 200 words)*

The use of depleted uranium (DU) munitions during Desert Storm resulted in a unique type of battlefield casualty, DU shrapnel wounds. The toxicity associated with embedded DU may differ significantly from other metals or other routes of uranium administration. This is a 6-month interim report of an 18-month study that is designed to assess the toxicity of implanted DU pellets. This study evaluates kidney, behavioral, and neural toxicity associated with intramuscularly implanted DU pellets (1-mm x 2-mm) and assesses tissues for histological changes and for uranium content. Rats were assigned to five experimental groups: 1) a non-implanted sham-surgical control group, 2) rats implanted with 20 tantalum (Ta) to control for fragment implantation, 3) rats implanted with low-dose DU (4 DU and 16 Ta pellets), 4) rats implanted with medium-dose DU (10 DU and 10 Ta pellets), and 5) rats implanted with high-dose DU (20 DU pellets). Uranium levels were high and dose-dependent in the kidney, urine, and bone. Despite high uranium levels in the kidney, no renal toxicity was evident. Between 23-26 weeks body weight in high-DU dose animals was significantly lower than controls. Unexpectedly, uranium was found in the brain of DU-implanted animals. No behavioral neurotoxicity was evident. Excitability of hippocampal neurons was reduced in the high DU dose animals at 6 months. These data suggest that at the 6-month time point, renal toxicity may be less of a hazard than anticipated.While these results indicate that toxicity is not evident at 6 months with exposure to embedded DU, there is a need to further investigate long-term effects in light of the high levels accumulated in some body tissues.

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